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Down-regulation of cladofulvin biosynthesis is required for biotrophic growth of *Cladosporium fulvum* on tomato

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SUMMARY

Fungal biotrophy is associated with a reduced capacity to produce potentially toxic secondary metabolites (SMs). Yet, the genome of the biotrophic plant pathogen *Cladosporium fulvum* contains many SM biosynthetic gene clusters, with several related to toxin production. These gene clusters are, however, poorly expressed during the colonization of tomato. The sole detectable SM produced by *C. fulvum* during *in vitro* growth is the anthraquinone cladofulvin. Although this pigment is not detected in infected leaves, cladofulvin biosynthetic genes are expressed throughout the pre-penetration phase and during conidiation at the end of the infection cycle, but are repressed during the biotrophic phase of tomato colonization. It has been suggested that the tight regulation of SM gene clusters is required for *C. fulvum* to behave as a biotrophic pathogen, whilst retaining potential fitness determinants for growth and survival outside its host. To address this hypothesis, we analysed the disease symptoms caused by mutant *C. fulvum* strains that do not produce or over-produce cladofulvin during the biotrophic growth phase. Non-producers infected tomato in a similar manner to the wild-type, suggesting that cladofulvin is not a virulence factor. In contrast, the cladofulvin over-producers caused strong necrosis and desiccation of tomato leaves, which, in turn, arrested conidiation. Consistent with the role of pigments in survival against abiotic stresses, cladofulvin protects conidia against UV light and low-temperature stress. Overall, this study demonstrates that the repression of cladofulvin production is required for *C. fulvum* to sustain its biotrophic lifestyle in tomato, whereas its production is important for survival outside its host.

Keywords: abiotic stress resistance, biotrophy, *Fulvia fulva*, natural product, regulation of secondary metabolism, virulence.

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INTRODUCTION

Secondary metabolites (SMs) are usually studied for their potent biological activities, which can be either beneficial (e.g. antibiotics, immune suppressors, anticholesterol drugs) or harmful (e.g. mycotoxins). Although their intrinsic functions are less frequently investigated, studies have shown that the production of bacterial, fungal and plant SMs is associated with competition and survival (Demain and Fang, 2000). Accordingly, the enormous chemical diversity of SMs is thought to result from adaptations of SM producers to different ecological niches (Osbourn, 2010). Filamentous fungi are important SM producers and sequenced fungal genomes have revealed that the Ascomycota have a particularly high potential for SM production (Collemare *et al.*, 2008; Ohm *et al.*, 2012). For instance, it has been estimated that the genomes of just four *Aspergillus* species, *A. fumigatus*, *A. nidulans*, *A. niger* and *A. oryzae*, collectively have the capacity to produce 226 different SMs (Inglis *et al.*, 2013).

Plant-pathogenic fungi are commonly classified into different lifestyles, which are the result of complex interactions with their hosts, with whom they have shared long co-evolutionary histories. Hemibiotrophic and necrotrophic fungal plant pathogens are well-known producers of SMs. During the colonization of their respective hosts, these fungi deploy SMs to promote disease. The necrotrophic plant-pathogenic fungi *Cercospora* spp. and *Elsinoë* spp., for instance, produce the non-specific toxins cercosporin and elsinochromes, respectively, which are photodynamic SMs that generate toxic reactive oxygen species (ROS) during host colonization (Daub and Hangarter, 1983). Indeed, mutants unable to produce these SMs show a significant reduction in virulence (Choquer *et al.*, 2005; Liao and Chung, 2008). The necrotrophic plant-pathogenic fungi *Cochliobolus* spp. and *Alternaria* spp. produce SMs that are host-specific toxins (Stergiopoulos *et al.*, 2012). These compounds are crucial in determining the fungal host range, with loss of SM production resulting in a loss of virulence (Wolpert *et al.*, 2002). In contrast, biotrophic fungal plant pathogens feed and complete their lifecycle on living host tissues. It is axiomatic that a biotrophic plant pathogen must limit the production of SMs that are toxic towards its host. In line with this, the non-obligate biotrophic maize pathogen *Ustilago maydis* contains

only three polyketide synthase (*PKS*) genes, which are unlikely to be functional, and only three non-ribosomal peptide synthetase (*NRPS*) genes, two of which are required for the production of siderophores (Bölker *et al.*, 2008; Winterberg *et al.*, 2009). Furthermore, the obligate biotrophic pathogens *Blumeria graminis* f. sp. *hordei*, *Melampsora larici-populina*, *Puccinia graminis* f. sp. *tritici* and *Puccinia triticina* have a highly reduced SM gene complement (Duplessis *et al.*, 2011; Spanu *et al.*, 2010). The last three species do not have any *PKS* genes and carry only a single *NRPS* gene, whereas *B. graminis* f. sp. *hordei* carries one *PKS* and one *NRPS* gene. These observations suggest that the loss of SM biosynthetic pathways is associated with the biotrophic lifestyle of these fungal pathogens (Collemare and Lebrun, 2011).

Genomic and transcriptomic analyses of *Cladosporium fulvum*, the biotrophic fungal pathogen responsible for tomato leaf mould disease, revealed 15 predicted SM gene clusters, including two that are related to the biosynthesis of the aforementioned cercosporin (*PKS7*) and elsinochrome (*PKS1*) toxins (Collemare *et al.*, 2014; de Wit *et al.*, 2012). The majority of *C. fulvum* SM gene clusters do not display evidence of inactivation, such as the accumulation of disruptive mutations (Collemare *et al.*, 2014). Yet, only the predicted siderophore synthetase *NPS9* gene and cladofulvin synthase gene *claG*/*PKS6* are significantly expressed in *C. fulvum* during growth on tomato, and in different *in vitro* culture conditions (Collemare *et al.*, 2014; Griffiths *et al.*, 2015, 2016). Because *claG* and *NPS9* are expressed in *C. fulvum* during the pre-penetration phase on the host leaf surface, but repressed during colonization of the apoplast surrounding tomato mesophyll cells, we hypothesized that the down-regulation and low expression levels of SM genes might be an alternative mechanism to the irreversible gene losses observed in other biotrophic pathogens (Collemare *et al.*, 2014). Similarly, many *C. fulvum* genes encoding predicted functional cell wall-degrading enzymes and carbohydrate-active enzymes are weakly expressed or silent during biotrophic growth (de Wit *et al.*, 2012). Consistent with our hypothesis, siderophore biosynthesis in the hemibiotrophic maize pathogen *Colletotrichum graminicola* is specifically down-regulated during biotrophic growth, which prevents the activation of host immune responses (Albarouki *et al.*, 2014).

The observed down-regulation of *claG* during tomato leaf colonization by *C. fulvum* represents a unique opportunity to experimentally assess the association between biotrophy and the repression of SM production. In this study, we manipulated the *claG* gene cluster in mutants to either prevent cladofulvin production or to cause its over-production during biotrophic growth on tomato. The resulting disease symptoms were compared to determine any link between cladofulvin production, biotrophic growth and virulence. In addition, we tested the possibility that cladofulvin protects *C. fulvum* against environmental stresses.

RESULTS

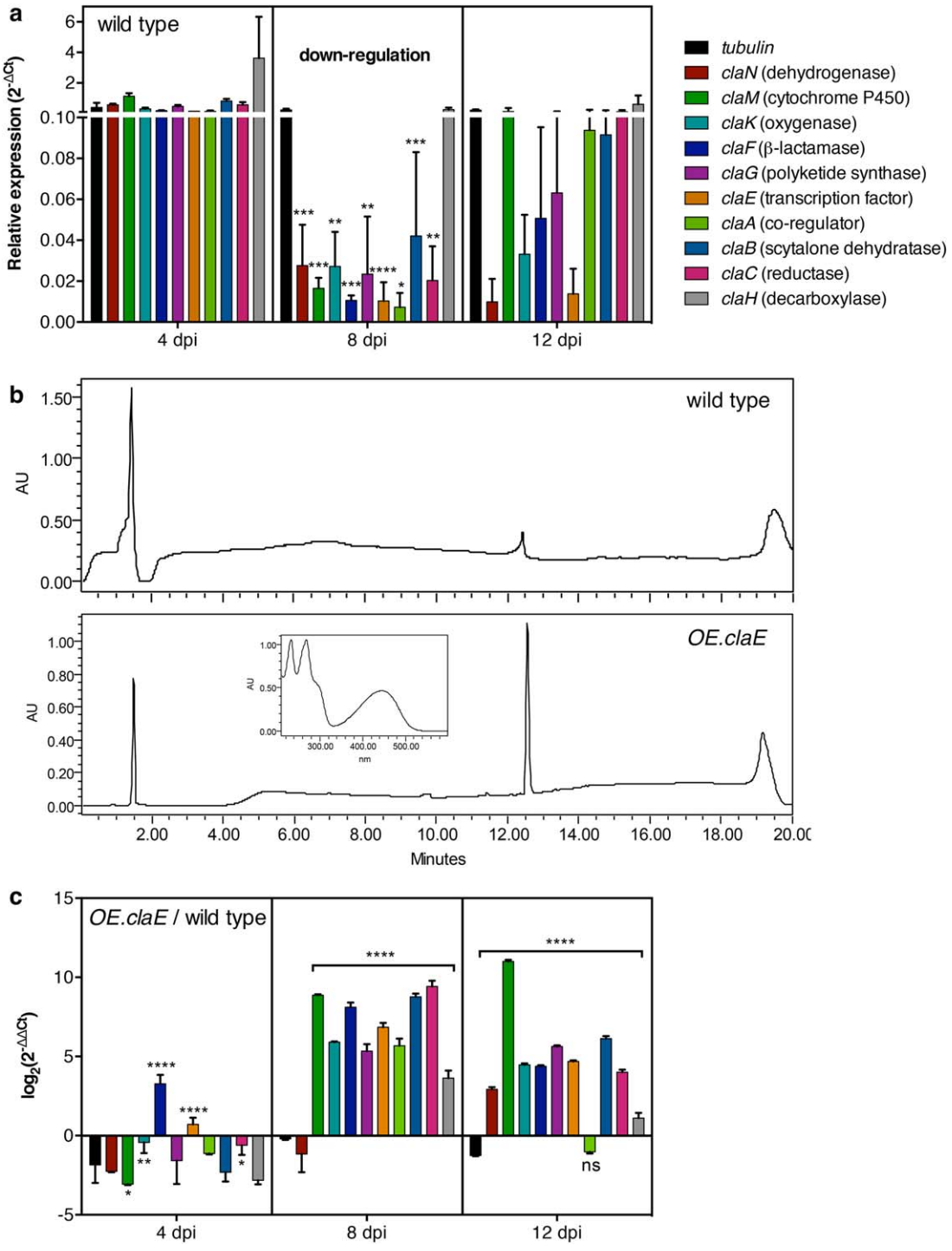
The cladofulvin gene cluster is down-regulated during the biotrophic growth of *C. fulvum* on tomato

The cladofulvin gene cluster is composed of nine co-regulated genes (*claA*, *claB*, *claC*, *claE*, *claF*, *claG*, *claK*, *claM* and *claN*), which encode seven enzymes involved in its biosynthesis, a predicted transcription factor (*claE*) and a putative co-regulator (*claA*) (Collemare *et al.*, 2014; Griffiths *et al.*, 2016). In addition, although not located at the same genomic locus, a decarboxylase-encoding gene (*claH*) is also co-regulated *in vitro* and involved in cladofulvin biosynthesis (Griffiths *et al.*, 2016). *claG* encodes the PKS that produces the first precursor in cladofulvin biosynthesis (Griffiths *et al.*, 2016). This gene is only expressed during the early and late stages of infection, but not during colonization of the apoplastic space surrounding leaf mesophyll cells (Collemare *et al.*, 2014). A similar expression pattern was obtained for other genes at the *claG* locus (Collemare *et al.*, 2014), but the predicted gene cluster in this study was not accurate and expression data were not available for all genes of the defined gene cluster (Griffiths *et al.*, 2016). The previous experiments needed to be repeated in order to confirm that the entire cladofulvin gene cluster is down-regulated *in planta*; the expression of each gene was measured by reverse transcription-quantitative real-time polymerase chain reaction (RT-qrtPCR) in a new time-course experiment. By 4 days post-inoculation (dpi), conidia had germinated and the runner hyphae were exploring the leaf surface in search of open stomata (Collemare *et al.*, 2014; de Wit *et al.*, 2012). At this stage, all cladofulvin genes exhibited expression levels similar to that of the endogenous control gene *tubulin* (Fig. 1a). At 8 dpi, when the fungus was colonizing the apoplastic space surrounding the mesophyll cells (Collemare *et al.*, 2014; de Wit *et al.*, 2012), all genes, except *claH*, were significantly down-regulated (Fig. 1a). Although not statistically significant, the expression of *claH* was reduced by 10-fold compared with its expression level at 4 dpi. The expression level of most genes started to increase at 12 dpi relative to 8 dpi, ranging from 1.2-fold for *claK* to 13-fold for *claA* (Fig. 1a). At 12 dpi, conidiophore formation and conidiation on the leaf surface had started (Collemare *et al.*, 2014; de Wit *et al.*, 2012). Our results confirm that all 10 genes involved in the cladofulvin biosynthetic pathway are co-regulated *in planta* and all are down-regulated during biotrophic growth within tomato leaves.

Cladofulvin is not required for the virulence of *C. fulvum* on tomato

Because the cladofulvin gene cluster is expressed prior to the penetration of stomata, it remained possible that cladofulvin is involved in virulence. Tomato plants were inoculated with the wild-type parental strain of *C. fulvum*, two independent Δ*clag* deletion mutants that are deficient in cladofulvin production only,

and an ectopic insertion transformant control. These genetically modified strains have been obtained previously and have been characterized *in vitro* (Griffiths *et al.*, 2016). The resulting disease symptoms were similar for all strains, with each producing visible signs of conidiation from 12 dpi onwards (Figs 2a, 3a,b). The growth of Δ*clag* deletion mutants *in planta* was similar to that of the wild-type and ectopic insertion transformant controls, except for the higher biomass of the Δ*clag-A* mutant at 12 dpi (Fig. 4). This observation suggests that the normal or slightly higher fungal biomass of these mutants at 12 dpi is not linked to *claG* deletion. Either way, these results are consistent with cladofulvin not being required for virulence.



Induction of the cladofulvin gene cluster and cladofulvin production during colonization of tomato triggers necrosis and leaf desiccation

The specific down-regulation of the cladofulvin gene cluster during leaf colonization suggests that cladofulvin might interfere with the biotrophic growth of *C. fulvum*. The presence of a conserved transcription factor gene (*claE*) inside the cladofulvin gene cluster allowed us to specifically activate this biosynthetic pathway, a strategy that has been proven to be successful in activating individual silent SM gene clusters in other fungi (Bergmann *et al.*, 2007; Cary *et al.*, 2015; Chiang *et al.*, 2010). For this purpose, we used the promoter of the effector gene, *Avr9*, which is highly induced under nitrogen starvation *in vitro* and *in planta* after the runner hyphae have penetrated the stomata of tomato leaves (Collemare *et al.*, 2014; Mesarich *et al.*, 2014; Van den Ackerveken *et al.*, 1994). By fusing the coding sequence of *claE* to the promoter region of *Avr9*, we aimed to induce cladofulvin biosynthesis once *C. fulvum* hyphae had entered the host. We first induced the expression of *ClaE in vitro* by growing the *OE.ClaE* transformant in the inductive Gamborg B5 medium without nitrogen (B5-N), which is a medium barely conducive for cladofulvin production in wild-type *C. fulvum* (Griffiths *et al.*, 2015). No obvious difference between the *OE.ClaE* transformant and wild-type was observed under this condition. Analysis of ethyl acetate extracts showed the appearance of a single major peak that corresponds to cladofulvin based on the retention time and UV spectrum (Fig. 1b; Griffiths *et al.*, 2015). This result shows that ClaE is an activator of the biosynthesis of cladofulvin only. During infection of tomato by the *OE.claE* transformant, the cladofulvin gene cluster is strongly induced at 8 and 12 dpi, apart from *claN* and *claA*, which show regular expression at 8 and 12 dpi, respectively (Fig. 1c). In contrast with the absence of cladofulvin during infection by *C. fulvum* wild-type (Collemare *et al.*, 2014) and consistent with gene cluster activation, cladofulvin could be detected as early as 4 dpi (0.011 ± 0.003 μg/mg leaf tissue; 0.014 ± 0.001 μg/mg leaf tissue at 8 dpi) and greatly accumulated by 12 dpi (0.268 ± 0.066 μg/mg leaf tissue). Remarkably, the *OE.claE* transformant triggered necrotic spots appearing between 8 and 10 dpi, followed by severe desiccation of the infected leaves from 12 dpi onwards (Fig. 2b). Also, in contrast with control strains, no conidiation on the leaf surface could be observed (Fig. 2a,b). Thus, activation of the cladofulvin gene cluster during colonization of tomato prevented *C. fulvum* from completing its lifecycle.

The *OE.claE* transformant was transformed with a plasmid carrying *GFP* to visualize leaf colonization using fluorescence confocal microscopy. The *OE.claE-GFP* transformant also caused necrosis and heavy desiccation of host tissue (Fig. 2b), but did not show any microscopic difference from the wild-type at 4 and 8 dpi (Fig. 3c). At 12 dpi, fluorescence was detected in hyphae within the necrotic lesions (Fig. 3c), confirming that hyphae were alive and physiologically normal. In contrast with all other controls, *OE.claE* and *OE.claE-GFP* transformants did not produce conidiophores with conidia on the leaf surface and no stromata were observed in the substomatal chambers (Fig. 3c). It is noteworthy that fungal hyphae in desiccated leaves remained viable for some time. Nine weeks after the onset of necrosis, infected material was recovered and placed on potato dextrose agar (PDA) supplemented with geneticin or on tissue paper saturated with water. Fluorescent conidiophores and conidia of the *OE.claE-GFP* transformant emerged after 72 h (Fig. 2c).

Cladofulvin plays a role in fitness and resistance to environmental stresses

Previously, no inhibitory activity could be found for cladofulvin against the bacteria *Pseudomonas fluorescens* and *Streptomyces coelicolor*, the necrotrophic fungus *Botrytis cinerea* and *Dicyma pulvinata*, a fungal mycoparasite of *C. fulvum* (Collemare *et al.*, 2014). Given that cladofulvin is not a virulence factor and is not produced during host colonization, the intrinsic biological function(s) of cladofulvin is probably confined to *ex planta* fitness and/or tolerance to environmental stresses.

When PDA plates were inoculated with an equal number of conidia of the wild-type, ectopic insertion transformants or Δ*clag* deletion mutants, and incubated for 7 days, the Δ*clag* strains produced about 50% fewer conidia compared with the control strains (Fig. 5a). This may be a consequence of the significantly lower germination rate of the Δ*clag* deletion mutants (1.3-fold difference; Fig. 5b) or reduced resilience of immature conidia that lack

Fig. 1 Relative expression of the cladofulvin gene cluster during infection of tomato by *Cladosporium fulvum*. Expression of the genes was measured by reverse transcription-quantitative real-time polymerase chain reaction (RT-qrtPCR) at 4, 8 and 12 days post-inoculation (dpi) and normalized to the expression of the *actin* gene using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Bars show the average of three independent biological repeats with standard deviation. (a) Relative gene expression during infection of susceptible Heinz tomato cultivar by wild-type *C. fulvum*. For each gene, each *in planta* time point was compared with the previous one using multiple *t*-tests (no consistent standard deviation; no correction for multiple comparisons; significance threshold $\alpha = 0.05$). (b) Diode array chromatograms (maximum plots) of ethyl acetate extracts from *C. fulvum* wild-type and *OE.claE* transformant grown in B5-N medium. Secondary metabolite detection was performed in the 200–800-nm wavelength range. The UV absorption spectrum of the compound detected in the main peak is shown and is diagnostic of cladofulvin (Griffiths *et al.*, 2015, 2016). The wild-type trace is reprinted from Griffiths *et al.* (2016), with permission from Elsevier. (c) Expression of the cladofulvin gene cluster during infection of tomato by the *OE.claE* transformant is compared with the *in planta* expression in the wild-type. For a given time point, each ratio was compared with the ratio for the *tubulin* control using a two-way analysis of variance (ANOVA) with *post-hoc* Dunnett's multiple comparisons test. Asterisks indicate significant differences (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$; ns, non-significant). The function of each gene according to Griffiths *et al.* (2016) is indicated in parentheses.

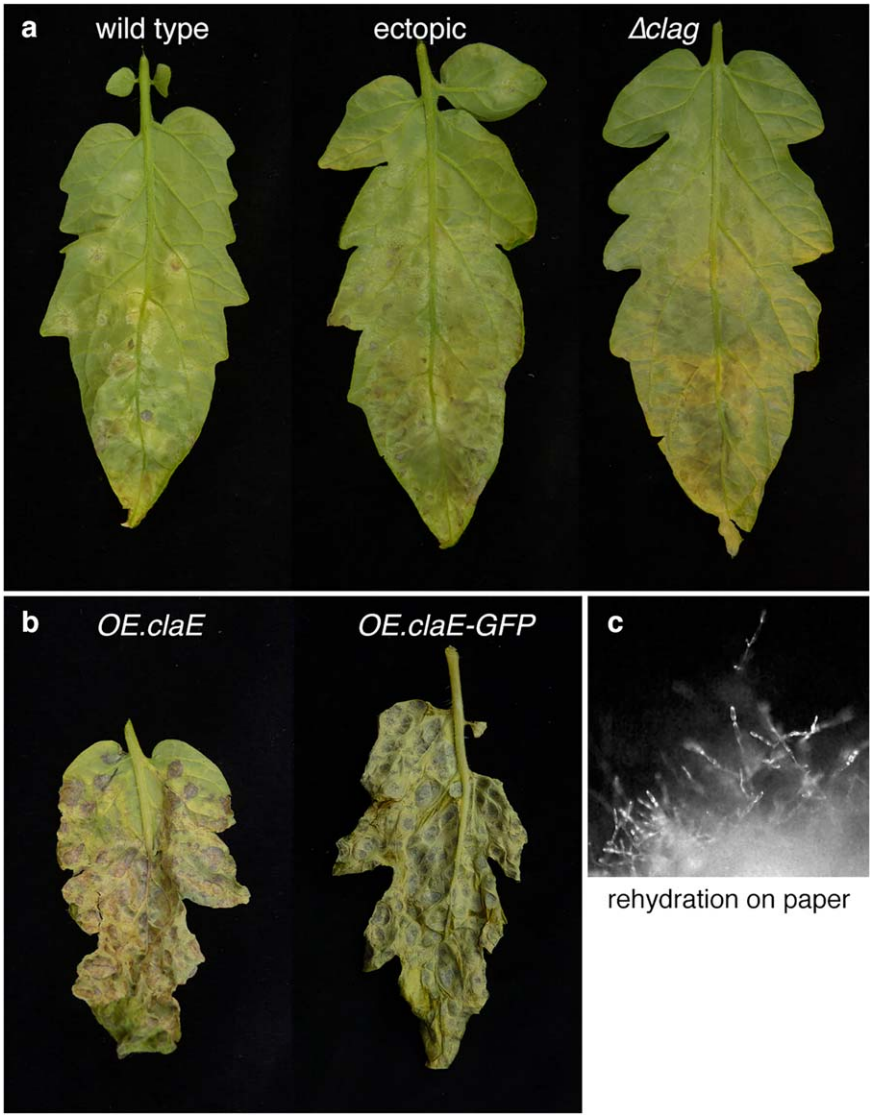


Fig. 2 Effect of cladofulvin production on virulence and biotrophy of *Cladosporium fulvum*. Leaves of the susceptible Heinz tomato cultivar were inoculated with conidial suspensions of the wild-type and transformants of *C. fulvum*. Symptoms were monitored daily and representative photographs were taken at 16 days post-inoculation. (a) Leaves inoculated with the wild-type, ectopic insertion transformant and Δ*clag* deletion mutant (not producing cladofulvin). (b) Leaf inoculated with the *OE.claE* and *OE.claE-GFP* transformants that over-express ClaE, the predicted local regulator of the cladofulvin gene cluster, specifically during host colonization. The Δ*clag* deletion mutants show typical leaf mould symptoms with conidiation on the leaf surface, whereas the *OE.claE* and *OE.claE-GFP* transformants induce necrosis, but do not produce conidia. (c) Three days after the rehydration of infected necrotic leaves on wet tissue paper, the *OE.claE-GFP* transformant produced green fluorescent protein (GFP) fluorescent conidia.

this pigment. However, such lower viability of mutant conidia did not alter their virulence in pathogenicity assays, but possibly could alter the spread of the disease in glasshouses.

After the growth of *C. fulvum* for 18 days in still liquid six-well plate cultures, mycelia had colonized the bottom of the well, but floating orange colonies had also grown on the surface (Fig. 5c). The submerged and floating colonies were separately recovered and both showed conidiation (Fig. 5c). However, high-performance liquid chromatography (HPLC) analysis of ethyl acetate extracts revealed a 17-fold difference between the concentration of cladofulvin produced by the green–black submerged mycelium (3.1 ± 1.1 μg/mg fungal biomass) and the floating orange colonies (51 ± 1.4 μg/mg fungal biomass), suggesting that cladofulvin is mainly produced during conidiation at the water–air interface.

Fungal conidia are protected from UV light through the synthesis of pigments. Indeed, phenolic compounds, such as anthraquinones and melanins, are photoprotective, strongly absorbing UV and visible light (Nguyen *et al.*, 2013). In order to measure the photoprotective effect of cladofulvin, the Δ*clag* mutant and control strains were irradiated for 180 s at 302 nm. The Δ*clag* mutant conidia were clearly more sensitive to UV light, with significantly fewer germlings produced after incubation for 48 h compared with the controls (4.2-fold difference; Fig. 5b). This result suggests a role in UV protection for cladofulvin.

High and low temperatures are also commonly encountered environmental stresses. To assess the protective role of cladofulvin against temperature stress, conidia from Δ*clag* deletion mutants, wild-type and ectopic insertion transformants were subjected to three cycles of freezing (−20 °C) and thawing at room temperature. Again, Δ*clag* mutant conidia were more sensitive with significantly fewer germlings produced after incubation for 48 h compared with the controls (4.4-fold difference; Fig. 5b). To assess the effect of

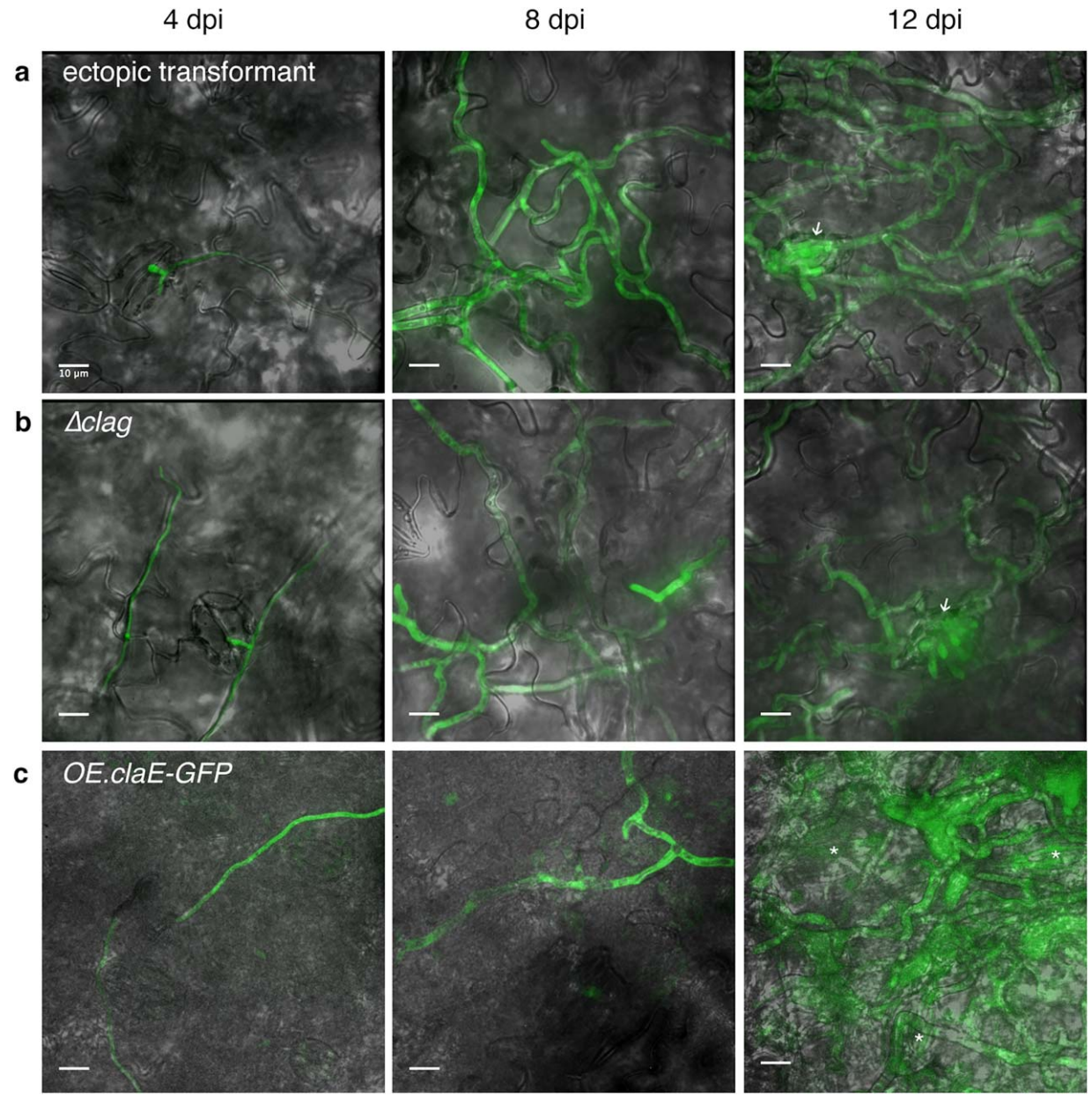


Fig. 3 Microscopic examination of the infection process of *Cladosporium fulvum* mutants. Transformed *C. fulvum* strains express the green fluorescent protein (GFP) constitutively. Fluorescence was observed at 4, 8 and 12 days post-inoculation (dpi) of tomato leaves using a confocal microscope equipped with a spinning disc. Stromata in substomatal chambers are indicated with arrows. (a) The ectopic insertion transformant showed a typical infection pattern with penetration at 4 dpi, colonization at 8 dpi and start of conidiation at 12 dpi. (b) The Δ*clag* deletion mutant, which does not produce cladofulvin, showed an infection pattern similar to that of the ectopic insertion transformant control. (c) The cladofulvin over-producing *OE.claE-GFP* transformant showed a normal penetration and colonization pattern; however, at 12 dpi, hyphae continued to grow, but no formation of stroma or production of conidia was observed. Asterisks indicate stomata with no visible stroma. Strong autofluorescence from plant cells was observed because of necrosis.

temperature on cladofulvin production, *C. fulvum* wild-type was incubated for 12 days in the dark at 22 °C, by which time the fungus had started to conidiate. Plates were then incubated at 10, 22 or 37 °C for 72 h. HPLC examination of ethyl acetate extracts showed that, relative to colonies incubated at the optimal growth temperature for *C. fulvum* (22 °C), cladofulvin production was highly induced at 10 °C, but significantly repressed at 37 °C (Fig. 5d). Taken together with the poor survival of Δ*clag* deletion mutants during freeze–thaw cycles, cladofulvin probably plays a role in protecting the conidia of *C. fulvum* against low temperatures.

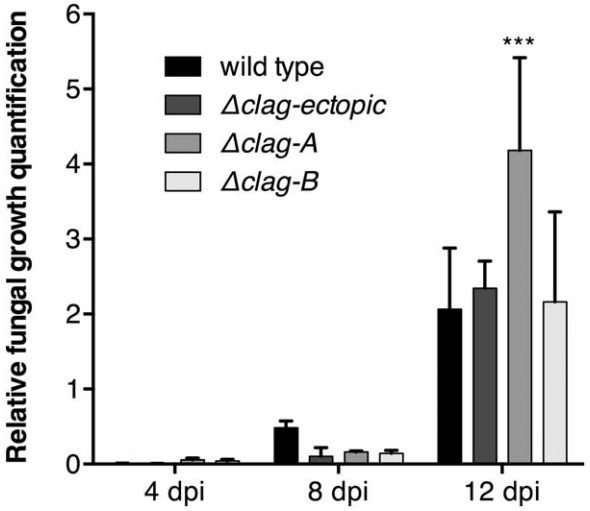


Fig. 4 Quantification of fungal growth during the infection of tomato by *Cladosporium fulvum* mutants. Total RNA was extracted at 4, 8 and 12 days post-inoculation (dpi). Fungal growth was estimated by reverse transcription-quantitative real-time polymerase chain reaction (RT-qrtPCR) for the wild-type, Δ*clag* deletion mutants and ectopic transformant. The expression of the fungal *actin* gene was normalized against the expression of the plant ribulose-1,5-bisphosphate carboxylase/oxygenase (*RUBISCO*) gene using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The wild-type and ectopic insertion transformants are shown as controls. Bars represent the mean and standard deviation of three biological replicates. A two-way analysis of variance (ANOVA) with *post-hoc* Fisher's least significant difference (LSD) multiple comparisons test was performed.

DISCUSSION

Down-regulation of SM gene clusters is a mechanism associated with biotrophic growth

Despite the potential of the biotrophic tomato pathogen *C. fulvum* to produce several SMs, cladofulvin is, so far, the only SM that has been isolated from this fungus (Agosti *et al.*, 1962; Collemare *et al.*, 2014; Griffiths *et al.*, 2015). When the fungus colonizes tomato leaves, the majority of its SM gene clusters are either silent or expressed so weakly that their relevance to virulence is questionable (Collemare *et al.*, 2014). Previous transcriptional profiling has indicated that genes at the *claG* locus are only expressed during the early and late stages of infection, but not during the colonization of mesophyll tissue (Collemare *et al.*, 2014). Here, we repeated this experiment and confirmed that the entire cladofulvin gene cluster (Collemare *et al.*, 2014; Griffiths *et al.*, 2016) is down-regulated during biotrophic growth. As *in planta* expression is mainly observed when conidia are present on the leaf surface, either as inoculum at the beginning of the infection or on conidiophores at the end of the infection cycle, one could argue that down-regulation of the cladofulvin gene cluster is related to fungal development only. However, our previous study on SM regulation by global regulators in *C. fulvum* has suggested that different regulatory networks control conidiation and cladofulvin production (Griffiths *et al.*, 2015). In addition, *C. fulvum* produces significant amounts of cladofulvin when grown in a minimal medium adjusted to pH 4, a condition that might resemble *in planta* conditions (Collemare *et al.*, 2014). The alternative hypothesis is that a host-derived signal triggers the observed down-regulation. Previously, we have found that saccharose, the prevailing disaccharide present in the apoplast of tomato (Joosten *et al.*, 1990), specifically represses cladofulvin production (Griffiths *et al.*, 2015). Thus, *in planta* repression of the *claG* gene cluster and cladofulvin production might be triggered by *C. fulvum* sensing saccharose as a signal of colonizing tomato leaves. It is likely that the sensing of saccharose regulates networks involved in the specific development of *C. fulvum* inside tomato leaves, networks that include the activation of effector genes (de Wit *et al.*, 2012) and down-regulation of the cladofulvin gene cluster.

When cladofulvin biosynthetic genes were induced in the *OE.claE* transformant during growth inside tomato leaves, we observed the formation of necrotic spots followed by severe desiccation of the infected leaves. However, *C. fulvum* could not complete its infection cycle because leaf desiccation prevented conidiophore formation and conidiation under conditions of normal relative humidity. As conidiation is essential to the long-term survival of the fungus, repression of cladofulvin production during tomato infection is required for the biotrophic lifestyle of *C. fulvum*.

Genome analyses of other biotrophic fungi have suggested that, among other genomic features, biotrophic growth is associated with the loss of SM biosynthetic pathways (Collemare and Lebrun, 2011). This has been observed in plant-pathogenic biotrophs, such as *B. graminis* f. sp. *hordei* and rust fungi (Duplessis *et al.*, 2011; Spanu *et al.*, 2010), but also in the symbionts *Tuber melanosporum* and *Laccaria bicolor*. These fungi possess only two *PKS* genes and one *NRPS* gene, and one *PKS* and one hybrid *PKS-NRPS* gene, respectively (Collemare and Lebrun, 2011; Martin *et al.*, 2008, 2010). *Laccaria bicolor* shows an expansion of a terpene cyclase gene family (TCs; 10 members), but it has been suggested that this large number of TCs is related to the symbiotic interaction between *L. bicolor* and its hosts, because the production of terpenes occurs more commonly in plants than in fungi (Collemare and Lebrun, 2011). The extensive loss of SM biosynthetic pathways in fungal biotrophs, such as *M. larici-populina*, *Puccinia* species and *T. melanosporum*, might have contributed to their evolution towards obligate biotrophy and symbiosis, respectively. Down-regulation of SM genes, as demonstrated here in *C. fulvum*, is proposed to be a mechanism, alongside other required adaptations, that would lead some plant-pathogenic fungi to develop a biotrophic lifestyle, whilst retaining potentially useful biosynthetic genes for its life outside the host.

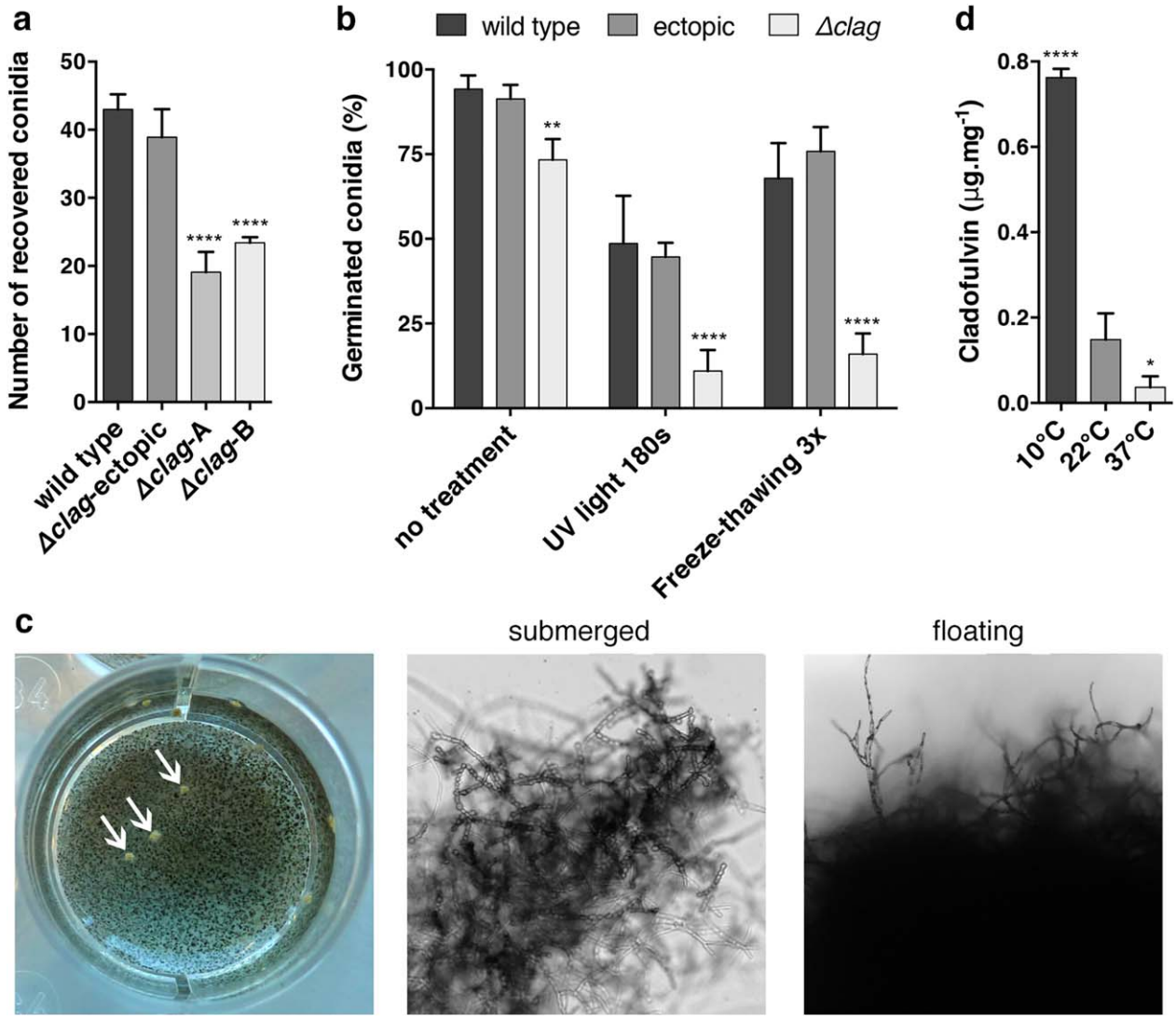


Fig. 5 Characterization of the biological function of cladofulvin. (a) Conidiation rate of *Cladosporium fulvum* wild-type, Δ*clag* deletion mutants and ectopic transformant. (b) The percentage of germination was determined for 100 conidia of each strain 48 h after performing no treatment, incubation under UV light (302 nm) for 180 s or three cycles of freeze–thaw. (c) *Cladosporium fulvum* grown in still liquid culture six-well plates colonized the bottom of the well to form a dark green mycelium, but orange floating colonies (a few are indicated by arrows) also developed. Both submerged and floating colonies differentiated conidia. The shape of submerged conidia is rounder, which could be caused by anaerobic stress. The orange colour of floating colonies is probably a result of the high production of cladofulvin. (d) Liquid chromatography quantification of cladofulvin produced by *C. fulvum* wild-type after incubation for 72 h at 10, 22 or 37 °C. Ethyl acetate extracts were analysed by high-performance liquid chromatography (HPLC) and the concentration of cladofulvin was determined using the response factor (RF) of pure cladofulvin as reference. For (b) and (d), an ordinary one-way analysis of variance (ANOVA) with a *post-hoc* Dunnett's multiple comparisons test was performed to compare with wild-type and 22 °C conditions, respectively. For (c), a two-way ANOVA with a *post-hoc* Dunnett's multiple comparisons test was performed to compare with the wild-type condition for each treatment. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$.

Cladofulvin is not a virulence factor, but triggers necrosis in the presence of *C. fulvum*

Using *C. fulvum* Δ*clag* mutants, we showed that both cladofulvin and its core biosynthetic gene *claG* are dispensable for virulence. Indeed, these mutant strains infected tomato leaves, and showed normal colonization and production of conidiophores bearing conidia.

In sharp contrast, symptoms caused by the *OE.claE* transformants were very different. They produced large amounts of cladofulvin, especially between 8 and 12 dpi, which was concomitant with the appearance of necrotic spots on tomato leaves. The hyphal growth of *C. fulvum* inside host tissues did not seem to be altered according to microscopic observations. This suggests that *C. fulvum* is able to grow as a saprobe inside necrotic tissues as

this fungus is not an obligate biotroph. It also shows that the induction of cladofulvin biosynthesis when it is not normally produced is not detrimental to the growth of *C. fulvum* hyphae. Later, strong desiccation of tomato leaves was observed under normal relative humidity, which prevented *C. fulvum* from producing conidia. How cladofulvin triggered these symptoms is unclear. In contrast with the necrosis induced in tomato leaves following infiltration of the phytotoxin dothistromin, infiltration of pure cladofulvin does not trigger any phenotype (Collemare *et al.*, 2014). Although cladofulvin localization is not known, one hypothesis could be that its target in tomato cells only becomes accessible after the alteration of tomato cell physiology by fungal hyphae. Another hypothesis is that the *OE.ClaE* transformant produces another toxic SM. However, *in vitro* activation of ClaE resulted only in the production of cladofulvin in large amounts, suggesting that it is unlikely that another SM is responsible for the observed necrosis. Alternatively, cladofulvin could affect the plant defence system. Siderophore production in the maize pathogen *C. graminicola* is specifically down-regulated during biotrophic growth (Albarouki *et al.*, 2014). Infiltration of the siderophore coprogen in maize leaves did not trigger any symptoms, but subsequent inoculation of *C. graminicola* resulted in strong induction of H_2O_2 production by plant cells at penetration sites (Albarouki *et al.*, 2014). It was suggested that coprogen primed the plant immune system to respond to infection by *C. graminicola* (Albarouki *et al.*, 2014). A similar mechanism could explain the appearance of necrotic spots only when cladofulvin and *C. fulvum* are present at the same time. The observed necrosis and desiccation might therefore represent a strong plant defence reaction, which requires further investigation.

Cladofulvin protects *C. fulvum* conidia from abiotic stresses

We have demonstrated that cladofulvin production *in planta* is strictly controlled, with the down-regulation of *claG* required for biotrophic growth of *C. fulvum* on tomato leaves. We have also shown that cladofulvin is not required for pathogenicity. The expression of the cladofulvin gene cluster during the pre-penetration phase and late stages of infection by *C. fulvum* suggests biological functions related to *C. fulvum* survival outside of its host.

Cladofulvin is not active against selected species of plants, bacteria and fungi, including *D. pulvinata*, a mycoparasite of *C. fulvum* (Collemare *et al.*, 2014), but is extremely toxic towards mammalian cell lines (Griffiths *et al.*, 2016). We hypothesized that it might protect *C. fulvum* conidia against pests, such as insects or fungivorous nematodes (Rohlfs *et al.*, 2007). However, Colorado potato beetles (*Leptinotarsa decemlineata*) and cabbage looper caterpillars (*Trichoplusia ni*) did not discriminate between tomato leaves inoculated with water or water containing a very high density of *C. fulvum* conidia (results not shown), suggesting that cladofulvin does not deter these polyphagous pests.

Instead of a role in competition with other organisms, the intrinsic biological function(s) of cladofulvin appears to be linked to the resistance of *C. fulvum* conidia to abiotic stress. We have shown that the germination of Δ*clag* is impaired compared with the wild-type, which might be caused by increased sensitivity to external stresses. Indeed, these Δ*clag* mutant conidia are significantly more sensitive to UV irradiation and cold stress. Altogether, our results suggest that cladofulvin is involved in protecting *C. fulvum* conidia, essential structures required for survival outside tomato in the environment and for transmission of the pathogen to new hosts. Certainly, the regulation of cladofulvin production has been fine tuned by both its biological functions and requirements for a biotrophic lifestyle.

It is difficult to draw conclusions about the relationship between SM gene loss and biotrophy considering that the supposedly lost ancestral gene clusters in these biotrophic fungi are not known. However, *C. fulvum* appears to be a good model for the study of this relationship because numerous silent gene clusters are present in its genome. Altogether, for this fungus, we have provided experimental proof that the repression of a fungal SM is necessary for a biotrophic plant pathogen to complete its infection cycle. Although pathway-specific regulators have been manipulated to activate SM gene clusters *in vitro*, to our knowledge, this is the first time this method has been applied to study SM production during a pathogen–host interaction. One interesting future challenge is to individually activate other silent SM gene clusters in *C. fulvum*, such as *PKS1* and *PKS7*, two gene clusters linked to the biosynthesis of phytotoxic perylenequinones.

EXPERIMENTAL PROCEDURES

Fungal strains used in this study

Cladosporium fulvum 0WU (de Wit *et al.*, 2012) was the parental strain used to perform transformation. The *C. fulvum* Δ*clag*-A, Δ*clag*-B deletion mutants and ectopic insertion transformants were created in a previous study (Griffiths *et al.*, 2016).

Generation of *OE.claE* and *OE.claE-GFP* transformants

The putative local regulator from the cladofulvin gene cluster, *claE*, was amplified by PCR using Phusion Flash High-Fidelity PCR Master Mix (Life Technologies, Carlsbad, CA) from *C. fulvum* genomic DNA with the primer pair *PacI_claE_Forward* (GCTACAGTTAATTAAATGTCCCTGTCACGCAGCGTGGCTG) and *NotI_claE_Reverse* (CATGTAGCGCGGCCGCTCACAGATTCTTCAGACGATC) (expected size, 1341 nucleotides). The *claE* amplicon was cloned into pFBTS3 (Griffiths *et al.*, 2015), a plasmid that contains the inducible promoter of the *Avr9* gene (Van den Ackerveken *et al.*, 1994). The *claE* amplicon and pFBTS3 were restricted using *Pac*I and *Not*I restriction enzymes (Fermentas Fast Digest, Waltham, MA), cleaned with Zymogen DNA Clean & Concentrator (BaseClear, Leiden, The Netherlands) and

ligated using T4 DNA polymerase (Promega, Madison, WI) to yield *pFBTS3-claE*. *Escherichia coli* DH5α cells were transformed using a standard heat-shock protocol and transformants were selected on Luria-Bertani (LB)-kanamycin agar (50 μg/mL). Plasmids were extracted from transformants and screened by restriction digest analysis using *Pac*I and *Not*I. A plasmid bearing the correct restriction pattern was sent to Macrogen (Amsterdam, the Netherlands) for sequencing of the insert. *Agrobacterium tumefaciens* AGL1 was transformed with *pFBTS3-claE* by electroporation, and plated on LB-kanamycin agar (50 μg/mL). One positive transformant was picked, verified and named AT-pFBTS3-claE. The plasmid was introduced into *C. fulvum* using *A. tumefaciens*-mediated transformation, as described previously (Ökmen *et al.*, 2013). Transformants were selected on PDA medium supplemented with hygromycin (100 μg/mL). Several transformants and wild-type *C. fulvum* were grown on potato dextrose broth (PDB; Oxoid, Altrincham, UK) and transferred to Gamborg B5 medium without nitrogen in order to induce the *Avr9* promoter (Van den Ackerveken *et al.*, 1994). Total RNA was extracted as described previously (Ökmen *et al.*, 2013) and cDNA synthesis was performed as described below. The induction of the cladofulvin biosynthetic cluster was confirmed by quantitative real-time RT-PCR using published primers (Griffiths *et al.*, 2016). One transformant showing the expected strong induction of cladofulvin biosynthetic genes was selected and named *C. fulvum OE.claE*.

Using the same methods, *A. tumefaciens* AGL1 was transformed with plasmid pRM254, which contains *GFP* and geneticin resistance genes (Mehrabi *et al.*, 2015), to yield AT-pRM254. The plasmid was introduced into the *C. fulvum OE.claE* transformant as described above. Transformants were selected on PDA medium supplemented with geneticin (100 μg/mL). One transformant was picked, screened for *GFP* fluorescence and named *C. fulvum OE.claE-GFP*.

Plant inoculation, determination of fungal growth and *in planta* gene expression

Inoculation of tomato with *C. fulvum* wild-type, deletion mutant and transformant strains was carried out according to a previously described method (Mesarich *et al.*, 2014). To determine fungal growth, the fourth composite leaf of infected tomato plants was harvested at 4, 8 and 12 dpi, and flash-frozen in liquid nitrogen. Samples were ground to a fine powder in liquid nitrogen, and total RNA was extracted from 100 mg of material using a Zymogen Direct-zol™ RNA MiniPrep kit (BaseClear) according to the manufacturer's recommended protocol. Synthesis of cDNA was performed using 100–2000 ng of total RNA and M-MLV reverse transcriptase (Promega), following the manufacturer's protocol. The remaining powder was weighed, and retained for SM extraction and analysis. To assess *C. fulvum* growth during infection, the *actin* gene of this fungus was targeted by quantitative real-time RT-PCR using the *Cf-actin_RT-qrtPCR_F/Cf-actin_RT-qrtPCR_R* primer pair (Mesarich *et al.*, 2014). For sample calibration, the *Solanum lycopersicum* gene encoding the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (*RUBISCO*) was targeted using the *Sl-rubisco_qrtPCR_F/Sl-rubisco_qrtPCR_R* primer pair (Mesarich *et al.*, 2014). The same cDNA samples were used to measure the expression of cladofulvin biosynthetic genes by quantitative real-time RT-PCR using previously reported methods and primers (Collemare *et al.*, 2014; Griffiths *et al.*, 2016). The results were analysed according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and are the average of three biological replicates.

SM isolation from plant leaf homogenates and UV-HPLC analysis

The powder that was retained for SM analysis was weighed and soaked in 20 mL of acetone at room temperature for 24 h. The sample was passed through a Schleicher & Schuell 595½ folded paper filter and the supernatant was dried under a nitrogen flow at ambient temperature. Acetonitrile (ACN; 2 mL) was added and the samples were left to soak at room temperature for 24 h. ACN was decanted into a 2-mL micro-centrifuge tube and stored at −20 °C for subsequent analysis by UV-HPLC. Prior to analysis, samples in ACN were centrifuged at 20 000 ***g*** for 5 min in a micro-centrifuge tube, and then transferred to a 1-mL clear glass shell vial (WAT025054c). HPLC analysis with a Waters Symmetry reverse phase C18 column (WAT046980) was carried out on a Waters 600S system (Waters, Milford, MA). The sample was eluted with a variable gradient of solvents (A) H_2O and (B) ACN (both containing 0.1% trifluoroacetic acid) at a flow rate of 1 mL/min. The following gradient was used: 0 min, A (95%); 10 min, A (10%); 12 min, A (10%); 15 min, A (0%); 16 min, A (95%); 20 min, A (95%). UV spectra were obtained using a 996-photodiode array detector. Metabolite concentrations were calculated by measuring the surface area of the respective peaks (254 nm) and divided by the response factor (RF) of cladofulvin (Griffiths *et al.*, 2015). All reagents and solvents were of analytical and HPLC grade, respectively. *In vitro* activation of the cladofulvin gene cluster in the *OE.claE* transformant and SM profiling were performed in Gamborg B5-N medium, as described previously (Griffiths *et al.*, 2015).

Microscopic examination of *GFP*-expressing strains

Imaging of infected tomato leaves was performed using a spinning disc confocal microscope (Nikon Ti microscope body (Shinagawa, Tokyo, Japan), Yokogawa CSUX1 scanner (Musashino, Tokyo, Japan), Photometrics Evolve camera (Tucson, AZ), Metamorph software (Molecular Devices, Sunnyvale, CA), 491-nm laser line; 60× oil 1.40NA objective). Z-stacks were acquired with an internal spacing of 0.5 μm. Screening of transformants and imaging of *C. fulvum OE.claE-GFP* on wet tissue paper and colonies grown in PDB were performed using a Nikon 90i epifluorescence microscope [GFP fluorescence was visualized using a GFP-B filter cube (EX 460-500, DM 505, BA 510-560)]. All images were processed using Fiji software (Schindelin *et al.*, 2012).

Quantification of conidiation

Six-well culture plates containing PDA were inoculated with 2000 conidia of wild-type *C. fulvum*, Δ*clag*-A and an ectopic insertion transformant control, and incubated for 10 days in the dark at 22 °C. Conidia were recovered by flooding each well with water and abrading the colony using an inoculating loop. The samples were centrifuged at 4000 ***g*** and then re-suspended in an equal volume of water for enumeration using a haemocytometer to give the total conidia recovered per well. The results are an average of three technical replicates.

Quantification of cladofulvin

When *C. fulvum* wild-type is grown in still PDB liquid culture in six-well plates, mycelia are formed at the bottom and floating colonies develop on the surface. From each sample, submerged and floating colonies were recovered separately and transferred to micro-centrifuge tubes and freeze-dried. One millilitre of ethyl acetate and 1 mL of water were added and the samples were incubated at room temperature for 30 min on a rotary shaker. After centrifugation at 13 000 ***g*** for 15 min, the organic phase was recovered, transferred to a clean glass vial and concentrated under a dry nitrogen flow. In a separate experiment, *C. fulvum* wild-type was grown on PDA plates for 12 days in the dark at 22 °C. Plates were transferred to 10, 22 or 37 °C for 72 h. Agar plugs containing entire colonies were transferred to a 50-mL tube. Ethyl acetate (25 mL) was added and the tubes were incubated at room temperature for 30 min on a rotary shaker. After centrifugation at 4000 ***g*** for 15 min, the organic phase was recovered, transferred to a clean glass vial and concentrated under a dry nitrogen flow. All samples were re-suspended in 1 mL ACN for UV-HPLC analysis as described above.

Biological activity assays

Germination assay: 20 μL of a *C. fulvum* conidial suspension (1×10^5/mL in water) were transferred to a glass microscope slide and covered with a glass coverslip. The slides were placed in a humidity chamber and incubated at 20 °C in the dark. Conidia and germlings were enumerated using a bright-field microscope at 48 h post-incubation.

Freeze–thaw assay: slides were prepared as above and then subjected to three rounds of freeze–thaw cycling. One cycle consisted of 10 min at −20 °C, followed by incubation at room temperature for 10 min. After treatment, slides were incubated for 48 h and processed as above.

UV survival assay: slides were prepared as above, except that treatment was applied prior to the addition of a glass coverslip. Uncovered slides were positioned 1 cm above a Bio-Rad (Hercules, CA) ChemiDoc XRS UV transilluminator and irradiated for 3 min at 302 nm. Then, a glass coverslip was added and the slides were processed as in the above described germination assay.

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